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The biliary excretion of enterokinase in rats

Studies in normal, chronic ethanol-maintained and cirrhotic rats

David A. W. GRANT, Peter A. JONES and John HERMON-TAYLOR
Department of Surgery, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.

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The excretion of catalytically active human or pig enterokinase in hepatic bile after intravenous administration to normal rats or rats that had been maintained on 20% (v/v) ethanol for 1 year showed similar kinetics to that described for other serum-derived bile proteins. The half-life in serum was 2.5 min or less, and most of the enzyme was excreted within 45 min of administration. This was maintained when up to six successive doses were given at 90 min intervals. The mean amount excreted per dose was independent of the dose number and varied from 0.8% to 2.1% in the normal animals and 1.2% to 2.0% in the chronic ethanol-maintained animals. When three doses of enzyme were given at 30 min intervals, the total amount of active enterokinase recovered in bile was dose-dependent and was consistently higher in the rats drinking 20% (v/v) ethanol. The serum half-life of enterokinase in rats made cirrhotic by inhalation of carbon tetrachloride vapour was extended to 6 min or more. The amount of active enzyme recovered in bile was at least 50% less than in weight-matched normal rats, and excretion was not complete 2 h after intravenous administration. The possible significance of these findings in liver and pancreatic disease is discussed.

The pathogenesis of acute necrotizing pancreatitis is characterized by the inappropriate activation of the digestive zymogens within the pancreatic acini. It has been suggested that this may be due to the reflux of bile and pancreatic juice, carrying enterokinase and active trypsin from the duodenum into the pancreatic duct (Durr, 1979). Both human (Grant & Hermon-Taylor, 1976) and pig (Baratti *et al.*, 1973) enterokinases have been isolated and characterized to be glycoproteins of approx. 300000 daltons, and in man the enzyme has been localized to the brush border of the enterocytes lining the duodenum and the first 10–20 cm of the jejunum (Hermon-Taylor *et al.*, 1977). We suggest that some forms of acute necrotizing pancreatitis, especially ethanol-induced, may be associated with the liberation of enterokinase into the blood as a result of damage to the mucosa of the proximal small intestine. Such an event is not unlikely, since the absorption of intact macromolecules such as horseradish peroxidase (Worthington *et al.*, 1978), albumin (Warshaw *et al.*, 1974) and immunoglobulin G (Hemmings & Williams, 1978) across both healthy and ethanol-damaged mucosa has been demonstrated.

In mice intravenous administration of purified human or partially purified mouse enterokinase was followed by a rapid clearance of the enzyme by the liver (Grant *et al.*, 1979). The receptor responsible for this was localized to the hepatocyte, but did not appear to be the same as that previously described for the clearance of asialoglycoproteins. A similar finding has been described for the clearance of bovine milk lipoprotein lipase from serum in rats (Wallinder *et al.*, 1979). In a subsequent report we showed that approx. 1% of intravenously administered enterokinase was excreted in its active form in the hepatic bile of healthy Wistar rats within 1 h of administration (Grant *et al.*, 1980). In man the presence of active enterokinase in gall-bladder bile would be potentially hazardous, since the anatomical and functional relationships may permit active reflux of bile into the pancreatic-duct system. Furthermore, if enterokinase is inappropriately liberated into portal blood from damaged duodenal mucosa the amount excreted in bile may be dependent on the functional state of the liver. The present paper compares the clearance and subsequent biliary excretion of intravenously administered human and pig enterokinase in normal

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rats with those maintained on 20% (v/v) ethanol for 1 year or which had carbon tetrachloride-induced cirrhosis.

Materials and methods

Enterokinase

Human and pig enterokinase were each purified to homogeneity as previously described (Grant *et al.*, 1978). Pig enterokinase was assayed in rat serum or bile by using α-*N*-[^{3}H]acetyltrypsinogen (Magee *et al.*, 1981); enzyme activity was detected by release of the radiolabelled activation peptide, and 1 unit of enzyme activity was defined as that amount of enzyme releasing 100 d.p.m./min at 30°C. The assay conditions were as follows: between 2.5 and 20 μl of serum or bile was diluted to 25 μl with normal serum or bile and mixed with 0.15 ml of 20 mM-Tris/20 mM-maleic acid that had been adjusted to pH 8 with NaOH. To this was added 25 μl of a soya-bean trypsin inhibitor stock solution (2 mg/ml) and 25 μl of α-*N*-[^{3}H]acetyltrypsinogen substrate (approx. 0.05 μCi). After incubation at 30°C for 90 min, radiolabelled peptide was separated from the substrate by the addition of 0.2 ml of 25% (w/v) trichloroacetic acid. The incubation mixtures were stored at 4°C overnight before centrifuging briefly to pellet the precipitate, and 0.1 ml of the supernatant was mixed with 4 ml of PCS scintillation fluid (Hopkin and Williams, Romford, Essex, U.K.) for radioactivity counting in an Intertechnique SL 3000 instrument. Human enterokinase was assayed either by using the radiolabelled natural substrate as described, or with the synthetic substrate glycyl-L-tetra-aspartyl-L-lysine β-naphthylamide (Grant & Hermon-Taylor, 1979); one unit of activity was defined as that amount of enyzme hydrolysing 1 pmol of substrate/h at 37°C. For rat serum and bile the final concentration of Zn^{2+} was lowered to 2.5 mM, compared with 5 mM previously described for mouse serum.

Animal studies

Ethanol administration. For this, 30 female Wistar rats weighing 180–200 g were allowed access *ab libitum* to standard rat chow, but the drinking water was replaced by 20% (v/v) ethanol. Daily liquid intake was approx. 10 ml/rat. The animals were maintained on this regime for 1 year before experimentation.

Experimental cirrhosis. The livers of male Wistar rats were made cirrhotic by the method of McLean *et al.* (1969). Briefly, rats with initial weight between 175 and 200 g drank water containing 0.5 g of sodium phenobarbitone/litre and received twice-weekly inhalations of carbon tetrachloride vapour until they were lightly anaesthetized. This was continued for 10 weeks, during which the animals tripled their body weight. Enterokinase-clearance studies were performed within 10 days of the final carbon tetrachloride inhalation.

Clearance of enterokinase from rat serum and excretion in hepatic bile. Weight-matched normal control rats and experimental animals were anaesthetized with 6 mg of pentobarbitone sodium/100 g body wt. The duodenum was exposed through an upper mid-line incision and the proximal common bile duct was cannulated with 0.5 mm-bore Teflon tubing. A catheter was introduced into the inferior vena cava via the femoral vein and positioned to allow free collection of blood samples over a 9 h period.

Rats which had been drinking 20% (v/v) ethanol for 1 year received up to six equal intravenous doses of human enterokinase at 90 min intervals. The doses of enzyme that the rats received were in the range 8–16 μg of enterokinase protein. Simultaneous weight-matched controls received the same amount and the same number of intravenous loads of human enterokinase as the experimental animals. Blood samples (0.3 ml) were taken 1, 2, 5, 10 and 20 min after administration of the first and last intravenous doses of the enzyme, and hepatic bile was collected as 15 min samples over the 9 h experimental period.

In another experiment, three rats which had been drinking 20% ethanol for 1 year, together with three weight-matched controls, received three equal intravenous doses of 12, 6.75 or 3.75 μg of human enterokinase at 30 min intervals. Blood and bile samples were collected as described above.

Rats with experimentally induced cirrhosis were tested as follows. Four animals, together with their weight-matched controls, received three equal intravenous doses of pig enterokinase at 2 h intervals. The amount of enzyme that each rat received was in the range 5–10 μg/dose. Blood and bile samples were collected as described above.

Histology was performed on all liver biopsies after fixing in 10% (v/v) formalin and staining with haematoxylin/eosin and periodic acid/Schiff stain alternately.

Results and discussion

It has been suggested that macromolecules may enter the bile directly from serum by passage across the epithelium of the peribiliary capillaries (Sternlieb, 1972). Other evidence suggests that passage of serum-derived components through the hepatocyte is essential for their transfer to bile (Mullock *et al.*, 1978). Some serum-derived proteins, such as amylase (Donaldson *et al.*, 1979) and desialylated glycoproteins (Thomas & Summers, 1978), are present in bile at 1% or less of their serum concentrations, whereas others, such as immuno-

globulin A, are major constituents of bile and only present in serum at low concentrations (Mullock *et al.*, 1978). Polymeric immunoglobulin A is removed from the circulation by a hepatocyte cell-surface receptor, secretory component, and is rapidly transferred in endocytic vesicles to the canalicular membrane, where it is secreted in bile as an immunoglobulin-A–secretory-component complex (Orlans *et al.*, 1978). Enterokinase seems to behave like the former class of serum-derived bile proteins, since it appeared only in low concentration in bile; the simultaneous administration of approx. 10^4-fold molar excess of human polymeric immunoglobulin A did not compete for the clearance of human enterokinase in mice, indicating that the enzyme did not bind to secretory component (D. A. W. Grant, unpublished work).

The livers of rats restricted to drinking 20% ethanol for 1 year did not show any obvious histological changes, and this method of ethanol administration did not alter the kinetics of enterokinase clearance by the hepatocytes. In both the test and control groups the serum half-life of human enterokinase was 2.5 min or less, and this was maintained from the first to the last dose. Excretion of enterokinase in hepatic bile was rapid in both groups, and with the higher doses active enzyme could be detected within 15 min of administration. Most of the enterokinase was excreted during the 15–30 min collection period, and excretion was complete after 1 h.

When enterokinase was administered for up to six doses at 90 min intervals, the mean amount excreted per dose appeared to be independent of the dose number or intravenous load and increased by no more than 0.5% in the ethanol-drinking rats compared with the amount excreted by the control animals (Table 1). The rate of bile secretion in the ethanol-drinking rats was 75% of that of the controls, but the amount of enterokinase excreted per unit volume of bile had increased. The excretion of most of the catalytically active enzyme within 45 min of administration in both the rats that had been drinking ethanol and the weight-matched control group was in close agreement with the time course described for the transfer of carcinoembryonic antigen (Thomas, 1980) and of ^{125}I-labelled asialoglycoproteins (Hubbard & Stukenbrok, 1979) and insulin (Bergeron *et al.*, 1979) from the sinusoidal surface of the hepatocyte to the canalicular membrane. Within 15 min of presentation to the sinusoidal surface of hepatocytes, ^{125}I-labelled asialoglycoproteins are transported, possibly within endocytic vesicles, to a liposome/Golgi-rich region located close to the bile canaliculi (Hubbard & Stukenbrok, 1979). During this time 90% of the ^{125}I label remains acid-precipitable and therefore associated with macromolecules. Similarities between the clearance of enterokinase and that of other hepatocyte-directed glycoproteins (Ashwell & Morell, 1974), which have been subsequently shown to follow an intracellular route to the bile canaliculi (Hubbard & Stukenbrok, 1979), would suggest that the active enzyme detected in bile followed an identical path. From our studies it was not clear whether biliary enterokinase was derived from a discrete endocytosed population that had by-passed the lysosomes to reach the canalicular membrane, or whether it represented enzyme that had resisted degradation or remained catalytically active despite partial digestion by lysosomal hydrolases. Both human and pig enterokinases are very heavily glycosylated enzymes, and this may protect or delay their degradation within the lysosomes by increasing their resistance to proteolytic attack.

A consistent difference in the clearance of enterokinase between the rats drinking ethanol and weight-matched controls was seen when the three doses of enterokinase were administered at 30 min intervals to coincide with the peak biliary excretion. The amount of enzyme excreted by the control rats remained at 1.6–1.8% of the total enterokinase load and was independent of the dose, which varied from 3.75 to 12 μg of enzyme (Fig. 1). By comparison, the total amount of enterokinase excreted by rats maintained on 20% ethanol for 1 year was 2.8% when three doses of 12 μg were administered, 2.5% when three doses of 6.75 μg were given and 1.9% when three doses of 3.75 μg were given (Fig. 1). The increased excretion of active enzyme in the test group may have been due to more enterokinase by-passing the lysosomes in endocytic vesicles or to an increase in the rate of discharge of lysosome vacuoles carrying enzymes into the bile.

Experimentally induced cirrhosis was confirmed both macroscopically and histologically in rats that had inhaled carbon tetrachloride vapour. At nec-

Table 1. *Total amount of active human enterokinase recovered per intravenous dose in rat hepatic bile*

The recoveries were expressed as percentages of the load, and represent the mean for the numbers of animals tested (shown in parentheses). For details see the Materials and methods section.

	Rats	
Dose no.	Chronic ethanol-maintained	Normal
1	1.2 (7)	0.8 (4)
2	2.0 (6)	1.5 (4)
3	1.5 (6)	1.2 (4)
4	1.7 (4)	1.4 (3)
5	2.0 (3)	2.1 (3)
6	1.6 (3)	1.6 (3)

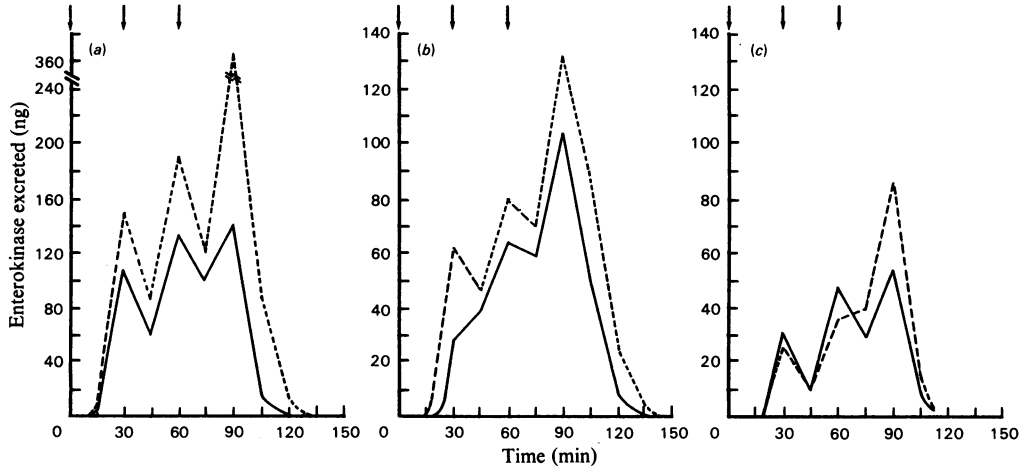

Fig. 1. *Excretion in bile of active human enterokinase after intravenous administration of three equal doses at 30 min intervals to rats drinking 20% ethanol for 1 year* (----) *or their weight-matched controls* (——)
Doses were (*a*) 3 × 12 μg, (*b*) 3 × 6.75 μg, (*c*) 3 × 3.75 μg. Arrows indicate time of administration. For details see the Materials and methods section.

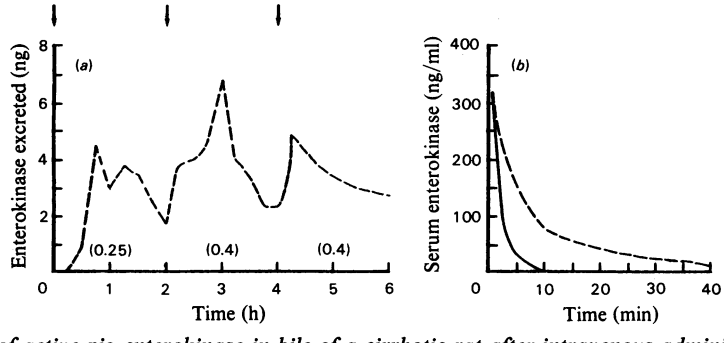

Fig. 2. (*a*) *Excretion of active pig enterokinase in bile of a cirrhotic rat after intravenous administration of three 7.5 μg doses at 2 h intervals (arrows), and (b) clearance of pig enterokinase from the circulation of a cirrhotic rat* (----) *and its weight-matched control* (——) *after intravenous injection of 7.5 μg* of enzyme
In (*a*), values in parentheses represent percentage of each intravenous load recovered in bile. For details see the Materials and methods section.

ropsy the livers were gritty and nodular, and some animals had ascites. Histological examination showed regeneration nodules separated by connective tissue and some bile-duct proliferation. The biliary excretion of enterokinase in thse animals was monitored with the pig enzyme rather than the human, but the results were equally applicable to enzyme from both species (Grant *et al.*, 1980). The mean serum half-life of enterokinase in rats with experimentally induced cirrhosis (⩾6 min) was more than double that of the weight-matched controls (⩽2.5 min) (Fig. 2). This was most probably due to a diminished blood supply to the sinusoidal surface of the hepatocytes as a consequence of a breakdown in the lobular structure of the liver, although a decrease in the number or affinity of the enterokinase receptors was also possible. A marked decrease in bile flow, to approx. 30% of that of weight-matched controls, was accompanied by a greater than 50% decrease in the amount of active enzyme excreted. The mean amount of enterokinase excreted by the four cirrhotic animals after each of the three doses was 0.25%, 0.4% and 0.4% respectively of the intravenous load, whereas in four control animals the equivalent values were 0.9%, 0.9% and 1.2%. Enterokinase activity could be detected in all the bile fractions collected in the 2 h intervals between doses, and excretion was not complete when the next dose was administered (Fig. 2). The small amount that appeared in bile may have been specifically trans-

ferred to the canalicular membrane without fusing with lysosomal elements and retained in this region until secreted with newly formed bile, or cirrhosis may have decreased or delayed the number of lysosomes emptying their contents into bile.

Ethanol has a direct toxic effect on the pancreas and causes profound ultrastructural and biochemical changes over the long term (Sarles *et al.*, 1971), but it is unlikely that these are the aetiological events leading to uncontrolled autoactivation of the pancreatic proteinases. From the animal studies reported here, we would suggest that superimposed on these changes is the handling by the liver of serum-derived enterokinase which has appeared in the circulation as a result of simultaneous damage to the intestinal mucosa. In man it would appear that ethanol-induced pancreatitis is more frequently associated with changes to the liver which are reversible, e.g. fatty liver, rather than with cirrhosis, which is irreversible (Seeff & Zimmerman, 1976). This would agree with our findings in the rat, where the excretion of enterokinase into bile was maintained or increased in animals drinking 20% ethanol for 1 year, but was attenuated in the cirrhotic animals. If this also occurs in man, then the presence of active enterokinase in gall-bladder bile could be the aetiological agent initiating pancreatitis of the type associated with alcoholism and may explain why the mean duration of ethanol consumption necessary for developing pancreatis appears to be shorter than that for developing alcoholic liver cirrhosis (Durbec *et al.*, 1979).

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